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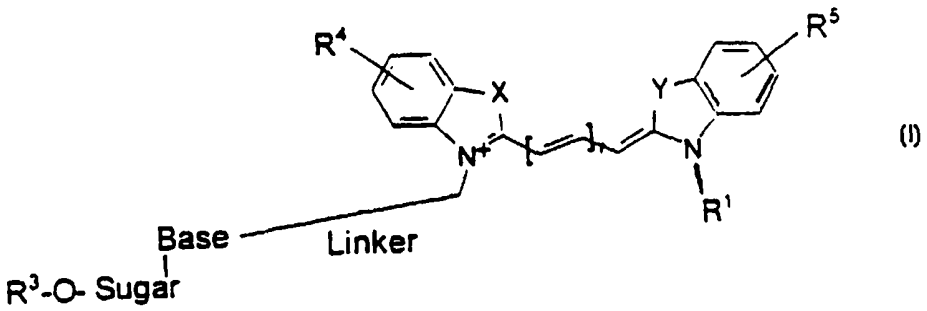

(I)

(57) Abstract

A chemical compound of formula: (I), wherein R^1 is selected from the group consisting of alkyl, aralkyl, and substituted alkyl groups; R^3 is selected from the group consisting of H, PO_3^{-2}; $P_2O_6^{-3}$; $P_3O_9^{-4}$, and α-thio phosphates (PSO_2^{-2}; $P_2SO_5^{-3}$; $P_3SO_8^{-4}$); and αBH_3^- phosphates ($P(BH_3)O_2^{-2}$, $P_2(BH_3)O_5^{-3}$, $P_3(BH_3)O_8^{-4}$); R^4 is selected from the group consisting of H, lower alkyl, acyl, $(CH_2)_pCOO(CH_2)_qCH_3$ wherein p is an integer from 0 to 4 and q is an integer from 0 to 4, and 5,6; 6,7; or 7,8- butadienyl, R^5 is selected from the group consisting of H, lower alkyl, acyl, $(CH_2)_pCOO(CH_2)_qCH_3$ wherein p is an integer from 0 to 4 and q is an integer from 0 to 4 and 5,6; 6,7; or 7,8- butadienyl; r is 1, 2 or 3 to form a second fused aromatic; X or Y are selected from the group consisting of O, S, $C(R^6)_2$, or $N(R^6)$, wherein R^6 is preferably CH_3 or a lower alkyl; and R^3-0 - Sugar-Base is a nucleoside or nucleotide is disclosed.

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NON-SULFONATED CYANINE DYES FOR LABELING NUCLEOSIDES AND NUCLEOTIDES

BACKGROUND OF THE INVENTION

Cyanine dyes have been described in the literature for many years[1,2], mainly for photographic purposes. In recent years, researchers have taken advantage of the excellent fluorescent properties of the carbocyanines to label biological molecules. Initial efforts were thwarted by the high background and/or quenching of fluorescence observed when the dyes were conjugated to proteins. The hydrophobic nature of the dyes caused them to aggregate in aqueous media or on the hydrophobic domains of proteins. Thus, the dyes, as described in the early literature, were not suitable for labeling.

Waggoner, et al.[3,4] disclosed the use of sulfonated derivatives of carbocyanines to label biological molecules. The sulfonate group was found to be effective at preventing aggregation, because of the repulsion of the negative charges between molecules. In some of the cited Waggoner disclosures, the importance of the sulfonate groups to the novelty and efficacy of the dye derivatives, which included nucleic acids, was emphasized.

US 5,556,959 discloses the use of carbocyanine phosphoramidites to label synthetic oligonucleotides. Due to the constraints of the automated systems used for DNA synthesis, the amidites had to be soluble in aprotic

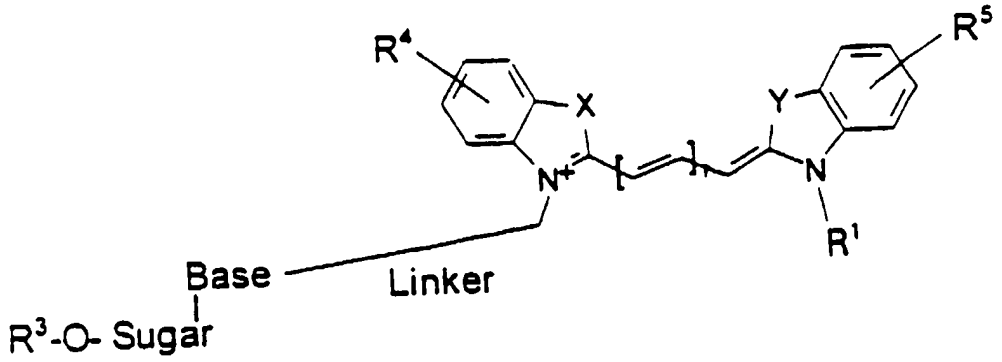
organic solvents. Sulfonated carbocyanines are insoluble in the solvents best suited for oligonucleotide synthesis. Therefore, the dye amidites described in US 5,556,959 lacked the sulfonate groups. Experiments showed that the amidites were soluble in the appropriate solvents, such as acetonitrile and dichloromethane, and labeled the oligonucleotides in high yield. The dye amidites and intermediates are easily and efficiently synthesized and purified.

Nucleoside triphosphates (NTPs) labeled with reporter groups have been in use for many years[5,6]. NTPs labeled with sulfonated carbocyanines have been reported in the scientific literature[7a], and are commercially available[7b]. However, synthesis of sulfonated cyanines is a difficult procedure, and the purity of the dye intermediates used in labeling is variable. The recommended shelf-life is short. Reagents for labeling are therefore expensive, as are the labeled NTPs derived from them.

Needed in the art of molecular biology is a nonsulfonated cyanine dye attached to a nucleotide or nucleoside.

BRIEF SUMMARY OF THE INVENTION

The present invention is a chemical compound of the following formula:



wherein R^1 is selected from the group consisting of alkyl, aralkyl, and substituted alkyl. Preferable R^1 substitutions include, but are not limited to, OR^2, $COOR^2$, NR^2R^2, SR^2, most preferably where R^2 is H, a removable protecting group, or a lower alkyl group. R^3 is H, PO_3^{-2}; $P_2O_6^{-3}$; $P_3O_9^{-4}$, α-thio phosphates, such as PSO_2^{-2}; $P_2SO_5^{-3}$; $P_3SO_8^{-4}$, and αBH_3^- phosphates, such as $P(BH_3)O_2^{-2}$, $P_2(BH_3)O_5^{-3}$, $P_3(BH_3)O_8^{-4}$. R^4 is selected from the group consisting of H, lower alkyl, acyl, and $(CH_2)_pCOO(CH_2)_qCH_3$, wherein p is an integer from 0 to 4 and q is an integer from 0 to 4. R^5 is selected from the group consisting of H, lower alkyl, acyl, and $(CH_2)_pCOO(CH_2)_qCH_3$ wherein p is an integer from 0 to 4 and q is an integer from 0 to 4. R^4 or R^5 may also be 5,6; 6,7; or 7,8-butadienyl (thus forming a second fused aromatic ring). r is 1, 2, or 3 and X and Y are O, S, $C(R^6)_2$, $N(R^6)$ (wherein R^6 is preferably CH_3 or a lower alkyl). R^3-O - Sugar - Base is a nucleotide or nucleoside.

It is an object of the present invention to provide a nucleotide or nucleoside attached to a nonsulfonated cyanine dye.

It is another object of this present invention to provide a nucleoside or nucleotide attached to a fluorescent label.

Other objects, advantages, and features of the present invention will become apparent after one has examined the specification, claims, and drawings of the present invention.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 is a schematic diagram of the synthesis of a cyanine dye-linked nucleoside originating with an amidite synthesis intermediate.

Fig. 2 is a schematic diagram of the synthesis of a cyanine dye-linked nucleotide.

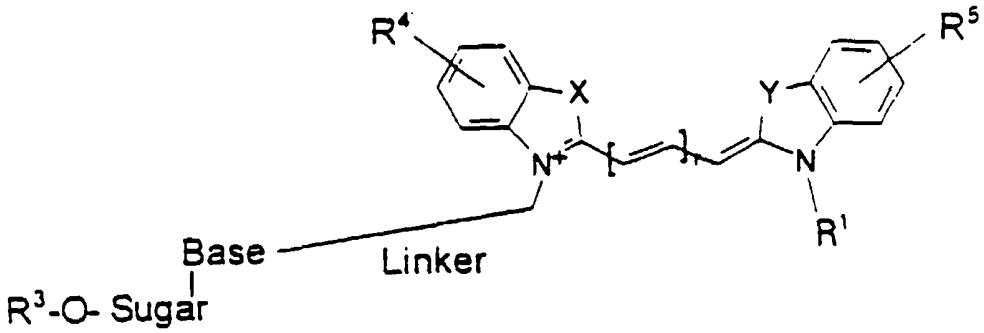
Fig. 3 is a diagram of indodicarbocyanine (IDC)-dCTP.

Fig. 4 is a diagram of alternative linkers.

Fig. 5 is a diagram of the general formula of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a chemical compound of the general formula



R^1 is selected from the group consisting of alkyl, aralkyl, and substituted alkyl chains. Preferably, the substitutions are OR^2, $COOR^2$, NR^2R^2, SR^2, where R^2 is preferably H, a removable protecting group such as trityl or acetyl, or a lower alkyl group (n= 1-4). In the examples below, we describe a compound of the present invention in which R is $(CH_2)_3OH$. Other preferred R groups are $(CH_2)_5COOH$, $(CH_2)_3NH_2$, and C_2H_5.

R^3 is either H, PO_3^{-2}, $P_2O_6^{-3}$, $P_3O_9^{-4}$, α-thio phosphates, such as PSO_2^{-2}, $P_2SO_5^{-3}$, $P_3SO_8^{-4}$, and αBH_3^- phosphates, such as $P(BH_3)O_2^{-2}$, $P_2(BH_3)O_5^{-3}$, or $P_3(BH_3)O_8^{-4}$.

R^4 is selected from the group consisting of H, lower alkyl, acyl, and $(CH_2)_pCOO(CH_2)_qCH_3$, wherein p is an integer from 0 to 4 and q is an integer from 0 to 4. R^5 is selected from the group consisting of H, lower alkyl, acyl, and $(CH_2)_pCOO(CH_2)_qCH_3$ wherein p is an integer from 0 to 4 and q is an integer from 0 to 4. R^4 or R^5 may also be 5,6; 6,7; or 7,8- butadienyl (thus forming a second

fused aromatic ring). r is 1, 2, or 3 and X and Y are O, S, $C(R^6)_2$, NR^6, wherein R^6 is preferably CH_3 or a lower alkyl (n=1-4), such as CH_2CH_3.

The butadienyl compounds are disclosed in 08/799,593 filed February 10, 1997, by Brush and Anderson, which hereby is incorporated by reference.

"Linker" is a combination of carbon, oxygen, nitrogen, and/or sulfur atoms in a chain that connects the dye through N1 to a position on the base. The linker may contain amide, ester, urea, thiourea, amine, ether, sulfide or disulfide bonds. The position on the base may be C^5 or C^6 of uracil, C^6 of thymine, N^4, C^5, or C^6 of cytosine, N^2, N^7, or C^8 of guanine, N^2, C^7, or C^8 of 7-deazaguanine, C^8 of hypoxanthine, C^7 or C^8 of 7-deazahypoxanthine, N^6 or C^8 of adenine, or N^6, C^7, or C^8 of 7-deazaadenine. Preferable linkers are listed below in the Examples (for example, propyl-O-PO_2-O-hexyl, propyl-O_2C-ethyl-CO, propyl-O_2C-ethyl-CONH-hexyl, and propyl-O_2C-ethyl-CONH-propynyl) and in Fig. 4. Preferable linkers are between 3 and 25 atoms in length.

Base, sugar and R^3 combine to form nucleotides and nucleosides known to one of skill in the art.

"Base" may be uracil, thymine, cytosine, guanine, 7-deazaguanine, hypoxanthine, 7-deazahypoxanthine, adenine, or 7-deazaadenine, 2,6-diaminopurine or other nitrogen-heterocycle bases, such as those described in reference 8 and references therein.

"Sugar" may be ribosyl, 2'-deoxyribosyl, 3'-deoxyribosyl, or 2',3'-dideoxyribosyl or 2'-oxabutyl, the sugar being preferably attached at N1 to the pyrimidines, and N9 to the purines and deazapurines.

"R^3-O-Sugar-Base" indicates that the R^3 group is preferably attached to the 5' oxygen of the sugar. In the case of the 2-oxabutyl "sugar," there is no 5' oxygen and the R^3 group would be attached to the 4' oxygen.

The Examples below disclose preferred methods of synthesis of the compound of the present invention.

In general, the synthesis of the compounds may be described as follows: The aromatic quaternary ammonium salt is prepared by alkylation of a 2-methyl indolenine, benzoxazole, or benzthiazole, or related benzo derivative. The alkylating agent contains a (protected) functional group which may be further derivatized. Two molecules of the resulting quaternary salt are condensed with one molecule of a protected unsaturated dialdehyde to yield a symmetrical cyanine. Alternatively, one molecule is condensed with a di-anil of an unsaturated dialdehyde. The product is then condensed with a different aromatic quaternary ammonium salt to give an unsymmetrical cyanine.

The functional group on the alkylating agent is then deprotected (if necessary) and derivatized to yield an activated group which is capable of reacting with a group on the nucleoside triphosphate. Many methods are known to the art which can be adapted to accomplish this.

The present invention is also a method of labelling a nucleic acid. Preferably, one would incorporate the compound described above into the nucleic acid chain in the same manner that one incorporates other nucleotides. In a most preferable form of the invention, one would then determine the nucleic acid sequence of the labelled nucleic acid molecule.

EXAMPLES

In General

The reaction schemes described below are general for several of the compounds made by the method. "NTP" signifies a nucleoside, or nucleoside mono-, di-, or triphosphate, bound through an amino group to the linker and dye. All the synthesized examples are triphosphates, as they are the most difficult, but also most useful, compounds to prepare.

The following abbreviations are used:

TABLE 1

	r =	X =
IMC = indomonocarbocyanine	1	$C(CH_3)_2$
IDC = indodicarbocyanine	2	$C(CH_3)_2$
ITC = indotricarbocyanine	3	$C(CH_3)_2$
BMC = benz(e)indomonocarbocyanine	1	$C(CH_3)_2$
BDC = benz(e)indodicarbocyanine	2	$C(CH_3)_2$
BTC = benz(e)indotricarbocyanine	3	$C(CH_3)_2$
OMC = benzoxazolemonocarbocyanine	1	O
ODC = benzoxazoledicarbocyanine	2	O
OTC = benzoxazoletricarbocyanine	3	O
TMC = benzthiazolemonocarbocyanine	1	S
TDC = benzthiazoledicarbocyanine	2	S
TTC = benzthiazoletricarbocyanine	3	S
NOMC = naphthoxazolemonocarbocyanine	1	O
NODC = naphthoxazoledicarbocyanine	2	O
NOTC = naphthoxazoletricarbocyanine	3	O
NTMC = naphththiazolemonocarbocyanine	1	S
NTDC = naphththiazoledicarbocyanine	2	S
NTTC = naphththiazoletricarbocyanine	3	S

Nucleoside	mono-phos	di-phos	tri-phos
A = adenine	(none)	(none)	(none)
rA = adenosine (ribo)	AMP	ADP	ATP
dA = 2'-deoxyadenosine	dAMP	dADP	dATP
ddA = 2',3'-dideoxyadenosine	ddAMP	ddADP	ddATP
aA = Acyclo (2'oxabutyl)	aAMP	aADP	aATP
Similarly, C=cytosine, cytidine; G= guanine, guanosine; c^7-G=7-deazaguanine T=thymine, thymidine; U=uracil, uridine			

Compounds prepared by the method described in Fig. 2 include:

IDC-rCTP

IDC-dCTP

IDC-ddCTP

ITC-ddCTP

ITC-ddATP

IDC-dATP

IMC-c7-ddGTP

OMC-ddCTP

IMC-ddCTP

A study using visible spectroscopy was done to determine whether the hydrophobic dyes aggregate in aqueous solution. The greater the shorter wavelength shoulder, the more aggregation is occurring. The concentration of indotricarbocyanine-ddATP, one of the most hydrophobic dye-nucleoside conjugates, was varied over a range from 0.6 to 80 μM. The ratio of 744 nm : 682 nm was observed (the wavelength maximum to the shorter wavelength at the shoulder of ITC spectrum). Variation was approximately ±5% over the entire range, compared to the ratio at a median absorption of A_{744} = 0.578 : A_{682} = 0.202, indicating very little, if any aggregation.

Experimental Procedures

Example 1

(Fig. 5, r=2, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O-PO_2-O-hexyl)-BASE; sugar = deoxyribosyl; base = adenine-N^6.)

1-3"-(N^6-hexyldeoxyadenosine, 5'-O-triphosphate)-propyl)phosphate)-1'-(3"'-hydroxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine

(ß-Cyanoethyl)
(1-(3"'-(1"'-propyl))-1'-(3"-(1"-(p-methoxytrityl) oxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine)
(6-N-trifluoroacetylaminohexyl) phosphate

The mono MMTr intermediate for the preparation of the corresponding amidite (1.13 g, 0.00145 mol), was treated with 6-N-trifluoroacetylaminohexyl-ß-cyanoethyl-N,N-diisopropylamino-phosphoramidite (1.4 g, 0.0065 mol) and 0.4 g (0.0056 mol) of tetrazole (in 2 mL of dry acetonitrile) in 7 mL of dry dichloromethane. The

reaction was stirred overnight, and then treated with 10 mL of 0.35 M iodine in a mixture of pyridine, water, and collidine. The solution was diluted with dichloromethane and extracted with aqueous bicarbonate and brine. The solution was dried and evaporated to leave the fully protected phosphotriester.

(1-(3'''-(1'''-propyl)-1'-(3"-oxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine) (6-aminohexyl) phosphate

The triester was dissolved in 50 mL of ethanol, to which 150 mL of 3:1 conc. ammonia/ethanol were added. After two hours at 60°C, the TFA and cyanoethyl protecting groups had been removed. The solvents were evaporated and the residue was dissolved in 50 mL of 7% trichloroacetic acid for one hour. The reaction mixture was neutralized by extraction with aqueous bicarbonate, dried, and evaporated. The residue was purified by C-18 NovaPak column chromatography with a 15 minute gradient of 40-100% acetonitrile in triethylammonium acetate, 0.1 M, pH 7.0. R_t = 7.8 minutes. The isolated material had the expected UV/visible spectrum, λ = 648 nm.

(1-(3'''-(1'''-propyl)-1'-(3"-oxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine) (6-aminohexyl N^6-deoxyadenosine, 5'-O-triphosphate) phosphate

The aminohexyl-derivatized dye above was dissolved in 500 μL of 0.1 M sodium borate, pH 9.2. To this was added 6-chloropurine-9-(1'-ß-deoxyriboside-5'-O-triphosphate). The reaction was stirred overnight at 60°C, after which time HPLC (C-18, 0-70% acetonitrile/TEAA) analysis showed a high percentage of the product. The main peak was isolated by prep C-18 HPLC, 10-50% acetonitrile/0.05 M ammonium phosphate, pH 7.2 over a 40 minute gradient. It was repurified twice by prep C-18 HPLC, 15-40% acetonitrile/0.05 M ammonium phosphate, pH 7.2 over a 40 minute gradient to a purity of >99%. The product was desalted on a C-18 cartridge

and stored in aqueous solution. The UV/visible spectrum showed the expected peaks at 648 nm for the dye and 268 nm for an N^6-derivatized adenine.

The material was compared with Cy5-29™-dATP, prepared by reaction of commercially obtained Cy5-29™-OSu (NHS ester) with N^6-aminohexyl-dATP. The UV/visible spectra were identical, and the automated sequencing results obtained on ALFexpress (Pharmacia Biotech) were comparable. The sequencing results demonstrate that there is no difference in the reaction of the unsulfonated material described here, and the Cy5™-29-labeled material, which bears two sulfonate groups.

Example 2

(Fig. 5, r=2, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CO)-BASE; sugar = deoxyribosyl; base = cytosine-N^4.)
1-3"-(N^4-6-amidohexyldeoxycytidine-5'-O-triphosphate)-succinoyloxypropyl)-1'-(3"'-hydroxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine (See Fig. 3)

(1-(3"'-(1"'-Propyloxysuccinic acid))-1'-(3"-(1"-(p-methoxytrityl)oxypropyl))-3,3,3', 3'-tetramethyl-indodicarbocyanine)

The mono MMTr intermediate for the preparation of the amidite (1 g, 0.00128 mol), was dissolved in 10 mL of pyridine and treated with 0.384 g (0.004 mol) succinic anhydride and 0.11 g 4-dimethylaminopyridine (0.0058 mol). The reaction was stirred for 4 hours at ambient temperature. Progress was monitored by C-18 HPLC on a 3 μm column at 80% acetonitrile/TEAA, isocratic, detected at 648 nm. After the addition of 1 mL of water, the reaction was evaporated to dryness. The residue was dissolved in dichloromethane and was extracted with aqueous bicarbonate and brine. After drying, the organic layer was evaporated to dryness.

(1-(3'''-(1'''-Propyloxysuccinic acid))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine)

The material from the previous reaction was dissolved in 30 mL of 80% acetic acid in water. After five hours at ambient temperature, the detritylation was complete, with no hydrolysis of the succinate ester. Progress was monitored by C-18 HPLC on a 3 μm column at 50% acetonitrile/TEAA, isocratic for 1 minute, then to 100% acetonitrile in 10 minutes, detected at 648 nm. The solution was evaporated and the residue dissolved in dichloromethane, extracted with aqueous bicarbonate three times, and brine. The solution was dried and evaporated to a blue powder.

(1-(3'''-(1'''-Propyloxysuccinic acid, N-hydroxysuccinimide ester))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine)

The dry solid was dissolved in 10 mL of dry dichloromethane, followed by 0.5 mL pyridine and 0.81 g (~3 eq) of O-trifluoroacetyl-N-hydroxysuccinimide. The reaction, monitored by C-18 HPLC on a 3 μm column at 50% acetonitrile/TEAA, isocratic for 1 minute, then to 100% acetonitrile in 10 minutes, detected at 648 nm, was over in 5 minutes. Dichloromethane was added to 30 mL and the solution was extracted with water three times, dried, and evaporated.

1-3"-(N⁴-6-Amidohexyldeoxycytidine-5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-hydroxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine

N⁴-(6-Aminohexyl)-dCTP was dissolved in 0.3 mL of 0.1 M sodium carbonate, pH 9.4. To this was added 200 μL of DMF, followed by 100 μL of DMF containing 10 mg of the aminolinker dye. The pH was readjusted to 9.4. The reaction was stirred for 1.5 hours at ambient temperature, at which time anion exchange HPLC (5-50% B in A over 30 minutes; A = 0.005 M sodium phosphate, pH

7.5 with 20% acetonitrile; B = A + 1 M NaCl) analysis showed a high percentage of the product at ~6 minutes. The main peak was repurified by C-18 HPLC, 5% for 2 minutes, then 5-60% acetonitrile/0.05 M ammonium phosphate, pH 7.2 over a 40 minute gradient. It was repurified twice prep C-18 HPLC, 15-40% acetonitrile/0.05 M ammonium phosphate, pH 7.2 over a 40 minute gradient to a purity of >99%. The product was desalted on a C-18 cartridge and stored in aqueous solution. The UV/visible spectrum showed the expected peaks at 648 nm for the dye and 276 nm for an N^4-derivatized cytosine.

The material was compared with Cy5-29™-dCTP, prepared by reaction of commercially obtained Cy5-29™-OSu (NHS ester) with N^4-aminohexyl-dCTP. The UV/visible spectra were identical, and the sequencing results obtained on ALFexpress (Pharmacia Biotech) were comparable. The sequencing results demonstrate that there is no difference in the reaction of the unsulfonated material described here, and the Cy5™-29-labeled material, which bears two sulfonate groups.

Example 3

(Fig. 5, r=2, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R' = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-hexyl)-BASE; sugar = ribosyl; base = cytosine-N^4.)

1-3"-((N⁴-6-Amidohexylcytidine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-hydroxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine

N^4-(6-Aminohexyl)-CTP (10 mg), prepared from diaminohexane and CTP by bisulfite catalysis, was dissolved in 200 μL sodium carbonate buffer, pH 9.5. 10 mg of the indodicarbocyanine-NHS ester (see Fig. 2, X = $C(CH_3)_2$, r = 2) was added in 50 μL of DMF. The pH was adjusted to 9.5 and the reaction was allowed to proceed for 2 hours. The product was isolated by NovaPak C18

HPLC (A = 0.05 M ammonium phosphate, pH 7.5, B = acetonitrile: 5% B for 2 minutes, 5-60% B for 40 minutes). The appropriate peaks were pooled and desalted on a C-18 cartridge. The UV/visible spectrum showed the expected peaks at 646 nm for the dye and 276 nm for an N^4-derivatized cytosine.

Example 4

(Fig. 5, r=2, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-hexyl)-BASE; sugar = dideoxyribosyl; base = cytosine-N^4.) **1-3"-((N⁴-6-Amidohexyl-2',3'-dideoxycytidine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3"'-hydroxypropyl))-3,3,3',3'-tetramethyl-indodicarbocyanine**

N^4-(6-Aminohexyl)-ddCTP, prepared from diaminohexane and 2',3'-dideoxy-CTP by bisulfite catalysis, (14 μmol) was dissolved in 1000 μL of 0.05 M sodium carbonate buffer, pH 9.5. To it was added 8 mg of the indodicarbocyanine-NHS ester (see Fig. 2, X = $C(CH_3)_2$, r = 2) in 150 μL of DMF and 150 μL water. The pH was adjusted to 9.3 and the reaction was allowed to proceed for 1 hour. The product was isolated by NovaPak C18 HPLC (A = 0.05 M ammonium phosphate, pH 7.2, B = acetonitrile: 0% B to 70% B for 40 minutes). The appropriate peaks were pooled and desalted on a C-18 cartridge. The UV/visible spectrum showed the expected peaks at 646 nm for the dye and 276 nm for an N^4-derivatized cytosine. Yield: 59% of material absorbing at 646 nm, by HPLC analysis. The material was incorporated by a DNA polymerase in a standard sequencing assay, terminating chain extension.

Example 5

(Fig. 5, r=3, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-hexyl)-BASE; sugar = dideoxyribosyl; base = cytosine-N^4.)

1-3"-((N⁴-6-Amidohexyl-2',3'-dideoxycytidine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3"'-hydroxypropyl))-3,3,3',3'-tetramethyl-indotricarbocyanine

1,1"-Bis-(3"-(1-hydroxypropyl))-3,3,3',3'-tetramethyl-indotricarbocyanine)

1-((3'-(1'-Acetoxypropyl))-2,3,3-trimethyl-(3H)-indolinium iodide (2 g) and 0.71 g glutacondialdehyde dianil were dissolved in a mixture of 40 mL acetic anhydride, 10 mL acetic acid, and 1 g of potassium acetate. The solution was refluxed for 20 minutes, at which time the ratio of A_{740} to A_{280} indicated that the reaction was complete. The solvents were evaporated and the residue was dissolved in dichloromethane, extracted three times with aqueous bicarbonate and once with brine, and evaporated. The residue was dissolved in 100 mL of methanol and 100 mL 4 M HCl were added. The reaction was stirred at ambient temperature overnight to complete the hydrolysis of the acetate esters. The solvents were evaporated and the residue was dissolved in dichloromethane, extracted three times with aqueous bicarbonate and once with brine, and evaporated. HPLC confirmed the conversion to the title compound, compared to the diacetyl. UV/vis: λ_{max} = 744 nm maximum

(1-(3"'-(1"'-Propyloxysuccinic acid))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethylindotricarbocyanine)

1,1"-Bis-(3"-(1-hydroxypropyl))-3,3,3',3'-tetramethyl-indotricarbocyanine) (0.5 g) was co-evaporated twice with dry pyridine, dissolved in 10 mL of pyridine and treated with 65 mg (1 eq.) of succinic anhydride and 0.055 g 4-dimethylaminopyridine. The reaction was stirred for 4 hours at ambient temperature. Progress was monitored by C-18 HPLC on a 4 μm column at 60% acetonitrile/TEAA, isocratic. After the addition of 1 mL of water, the reaction was evaporated to dryness.

The residue was dissolved in 10 mL dichloromethane, extracted with water, and dried. After drying, the organic layer was evaporated to dryness. The residue was dissolved in 10% acetonitrile in 1 M TEAA, pH 7 and purified on a prep HPLC on a NovaPak C18 cartridge with a gradient of 0-70% acetonitrile/0.1 M TEAA, pH 7. Yield: 30 mg.

(1-(3'''-(1'''-Propyloxysuccinic acid, N-hydroxysuccinimide ester))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethylindotricarbocyanine)

(1-(3'''-(1'''-Propyloxysuccinic acid))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethyl-indotricarbocyanine) was dried by co-evaporation twice with dichloromethane, then dissolved in 1 mL of dry dichloromethane and 0.05 mL pyridine. O-Trifluoroacetyl-N-hydroxysuccinimide (0.025 g) was added and the reaction was stirred. The reaction, monitored by C-18 HPLC on a 4 μm column with a gradient of 0-75% acetonitrile/TEAA, pH 7, detected at 648 nm, was over in 5 minutes. Dichloromethane was added to 30 mL and the solution was extracted with water three times, dried, and evaporated.
UV/vis: λ_{max} = 744 nm; yield: 34 mg at 80% purity.

1-3"-((N⁴-6-Amidohexyl-2',3'-dideoxycytidine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-hydroxypropyl))-3,3,3',3'-tetramethyl-indotricarbocyanine

N⁴-(6-Aminohexyl)-ddCTP, prepared from diaminohexane and 2',3'-dideoxy-CTP by bisulfite catalysis, (6 mg, 10 μmol) was dissolved in 700 μL of 0.07 M sodium carbonate buffer, pH 9.5. To it was added 5 mg of the indotricarbocyanine-NHS ester (Fig. 2, X = $C(CH_3)_2$, r = 3) in 100 μL of DMF and 100 μL water. The pH was adjusted to 9.3 and the reaction was allowed to proceed for 45 minutes. The product was isolated by NovaPak C18 HPLC (A = 0.05 M ammonium phosphate, pH 7.2, B = acetonitrile: 0%

B to 70% B for 40 minutes). The appropriate peaks were pooled and desalted on a C-18 cartridge. The UV/visible spectrum showed the expected peaks at 744 nm for the dye and 274 nm for an N^4-derivatized cytosine. Yield: 1.5 μmol. The material was incorporated by a DNA polymerase in a standard sequencing assay, terminating chain extension.

Example 6

(Fig. 5, r=3, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-hexyl)-BASE; sugar = deoxyribosyl; base = cytosine-N^4.)

1-3"-((N⁴-6-Amidohexyl-2'-deoxycytidine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-hydroxypropyl))-3,3,3',3'-tetramethylindotricarbocyanine

N^4-(6-Aminohexyl)-dCTP, prepared from diaminohexane and 2'-deoxy-CTP by bisulfite catalysis, (1 mg) was dissolved in 1000 μL of 0.1 M sodium carbonate buffer, pH 9.5. To it was added 1 mg of the indotricarbocyanine-NHS ester (Fig. 3, X = $C(CH_3)_2$, r = 3) in 50 μL of DMF and 50 μL water. The pH was adjusted to 9.3 and the reaction was allowed to proceed for 45 minutes. The product was isolated by NovaPak C18 HPLC (A = 0.05 M ammonium phosphate, pH 7.2, B = acetonitrile: 0% B to 70% B for 40 minutes). The appropriate peaks were pooled and desalted on a C-18 cartridge. The UV/visible spectrum showed the expected peaks at 744 nm for the dye and 274 nm for an N^4-derivatized cytosine. Yield: 50% of material absorbing at 744 nm is product, by HPLC analysis.

Example 7

(Fig. 5, r=3, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-hexyl)-BASE; sugar = dideoxyribosyl; base = adenine-N^6.)

1-3"-((N^6-6-Amidohexyl-2',3'-dideoxyadenosine, 5'-O-triphosphate)-succinoyloxypropyl) -1'-(3'''-hydroxypropyl))-3,3,3',3'-tetramethyl-indotricarbocyanine

N^6-(6-Aminohexyl)-ddATP, prepared by reaction of diaminohexane and 6-chloropurine-2',3'-dideoxyriboside-5'-O-triphosphate (10 μmol) was dissolved in 500 μL of 0.08 M sodium carbonate buffer, pH 9.5. To it was added 3 mg of the indotricarbocyanine-NHS ester (Fig. 3, X = $C(CH_3)_2$, r = 3) in 75 μL of DMF and 75 μL water. The pH was adjusted to 9.3 and the reaction was allowed to proceed for 2 hours. The product was isolated by NovaPak C18 HPLC (A = 0.05 M ammonium phosphate, pH 7.2, B = acetonitrile: 0% B to 70% B for 40 minutes). The appropriate peaks were pooled, the acetonitrile evaporated, and the product stored in ammonium phosphate solution. The UV/visible spectrum showed the expected peaks at 746 nm for the dye and 271 nm for an N^6-substituted adenine. Yield: 48 nmol.

Example 8

(Fig. 5, r=1, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-propynyl)-BASE; sugar = dideoxyribosyl; base = 7-deazaguanine-C^7.)

1-3"-((7-(3-Amidopropynyl-2',3'-dideoxy-7-deazaguanosine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-hydroxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine

(1-(3'''-(1'''-Propyloxysuccinic acid))-1'-(3"-(1"-(p-methoxytrityl)oxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine)

The mono MMTr intermediate from the preparation of the IMC amidite (0.2 g) was co-evaporated twice with dry pyridine, dissolved in 2 mL of pyridine, and treated with 0.077 g succinic anhydride and 0.022 g 4-dimethylaminopyridine. The reaction was stirred for 2

hours at ambient temperature. Progress was monitored by C-18 HPLC on a 3 μm column at 60% acetonitrile/TEAA, isocratic. After the addition of 0.2 mL of water, the reaction was evaporated to dryness. The residue was dissolved in dichloromethane and was extracted with aqueous bicarbonate and brine. After drying, the organic layer was evaporated to dryness. UV/vis: : λ_{max} = 550 nm.

(1-(3'''-(1'''-Propyloxysuccinic acid))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine)

The material from the previous reaction was dissolved in 10 mL of 80% acetic acid in water. After three hours at ambient temperature, the detritylation was complete, with no hydrolysis of the succinate ester. Progress was monitored by C-18 HPLC. The solution was evaporated and the residue dissolved in dichloromethane, extracted with aqueous bicarbonate three times, and brine. The solution was dried and evaporated. Yield 130 mg. UV/vis: λ_{max} = 550 nm.

(1-(3'''-(1'''-Propyloxysuccinic acid, N-hydroxysuccinimide ester))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine)

The dry solid co-evaporated twice with dry pyridine, and was dissolved in 2 mL of dry dichloromethane, followed by 0.1 mL pyridine and 0.2 g (~3 eq) of O-trifluoroacetyl-N-hydroxysuccinimide. The reaction, monitored by C-18 HPLC, was over in 5 minutes. Dichloromethane was added and the solution was extracted with water three times, dried, and evaporated. HPLC analysis (10-90% acetonitrile in 0.1 M TEAA, pH 7) showed the material to be about 85% pure. Yield 180 mg. UV/vis: λ_{max} = 550 nm.

1-3"-((7-(3-Amidopropynyl-2',3'-dideoxy-7-deazaguanosine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-

hydroxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine

7-(3-Aminopropynyl)-7-deaza-2',3'-ddGTP (0.25 μmol), obtained from NEN/DuPont, was dissolved in 0.1 M aqueous carbonate buffer, pH 9.5. (1-(3'''-(1'''-Propyloxysuccinic acid, N-hydroxysuccinimide ester))-1'-(3"-(1"-hydroxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine) (1 mg) was added in 50 μL each of DMF and water. The pH was adjusted to 9.2. After 1 hour the reaction was quenched by the addition of NaH_2PO_4 to pH 6.7. The product was purified on C-18 HPLC with acetonitrile and ammonium phosphate, pH 6. The appropriate peaks were pooled, the acetonitrile evaporated, and the product stored in ammonium phosphate solution. Yield: 29 nmol. UV/vis: λ_{max} = 550 nm.

Example 9

(Fig. 5, r=1, X = $C(CH_3)_2$, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-hexyl)-BASE; sugar = deoxyribosyl; base = cytosine-N^4.)

1-3"-((N⁴-(6-Amidohexyl-2'-deoxycytidine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-hydroxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine

N^4-(6-Aminohexyl)-dCTP (1 mg), prepared from diaminohexane and 2'-deoxy-CTP by bisulfite catalysis, was dissolved in 0.1 M aqueous carbonate buffer, pH 9.5. (1-(3-(1'''-propyloxysuccinic acid, N-hydroxysuccinimide ester))-1'-(3"-(1"- hydroxypropyl))-3,3,3',3'-tetramethyl-indomonocarbocyanine) (1 mg) was added in 50 μL each of DMF and water. The pH was adjusted to 9.2. After 1 hour the presence of the product was confirmed by HPLC by comparison to similar compounds. UV/vis: λ_{max} = 550 nm.

Example 10

(Fig. 5, r=1, X = O, R^4=R^5= H, R=$(CH_2)_3OH$, R^3 = 5'-O-triphosphate, linker = DYE-(propyl-O_2C-ethyl-CONH-hexyl)-BASE; sugar = dideoxyribosyl; base = cytosine-N^4.)

1-3"-((N⁴-6-Amidohexyl-2',3'-dideoxycytidine, 5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'"-hydroxypropyl))-benzoxazolmonocarbocyanine

1-((3'-(1'-Acetoxypropyl))-benzoxazolium iodide

2-Methylbenzoxaole (7.0 g 0.053 mol) and 13.2 g (0.03 mol) of 3-iodopropyl acetate were heated together at 100-110°C for 16 hours. The mixture was crystallized to a granular powder by trituration in ethyl acetate. It was filtered and dried by washing with ether. Yield: 15.1 g.

1,1"-Bis-(3"-(1-acetoxypropyl))-benzoxazolmonocarbocyanine

1-((3'-(1'-Acetoxypropyl))-benzoxazolium iodide (5 g, 0.014 mol) and 4.5 g of triethylorthoformate were dissolved in 100 mL dry pyridine. The solution was refluxed for three hours. The solvents were evaporated and the residue crystallized from ethyl acetate. Yield: 4.9 g. UV/vis: λ_{max} = 484 nm.

(1-(3"-(1"-acetoxypropyl)-1"-(3'"-(1'"-hydroxypropyl))-benzoxazolmonocarbocyanine

1-((3'-(1'-Acetoxypropyl))-benzoxazolium iodide (1 g) was stirred in a mixture of 20 mL of 4 N HCl and 20 mL methanol for 1 hour, then was rotovaped to dryness. The residue was dissolved in dichloromethane and extracted with water. The water was back-extracted three times with dichloromethane. The organic layers were combined, dried, and evaporated. The material was purified by C18 prep HPLC on a 25 x 200 mm Novapak cartridge using a 10-65% acetonitrile/TEAA gradient. The fractions enriched in the mono-acetyl derivative were pooled and evaporated to dryness.

(1-(3'''-(1'''-Propyloxysuccinic acid))-1'-(3'''-(1'''-acetoxypropyl))-benzoxazolmonocarbocyanine

The mono-acetyl derivative (175 mg) was dried by co-evaporation three times with dry acetonitrile and dissolved in 1 mL of dry pyridine. Succinic anhydride and DMAP were added and the reaction proceeded for 2 hours. The reaction was quenched with 1 mL water and the product purified by C18 prep HPLC on a 25 x 100 mm Novapak cartridge using a 0-80% acetonitrile/TEAA gradient. Yield: 22 mg.

(1-(3'''-(1'''-Propyloxysuccinic acid N-hydroxysuccinimide ester))-1'-(3'''-(1'''-acetoxypropyl))-benzoxazolmonocarbocyanine

The succinate derivative (22 mg) was dried by co-evaporation two times with dry pyridine and dissolved in 1 mL of dry dichloromethane with 50 μL of dry pyridine. O-Trifluoroacetyl-N-hydroxysuccinimide (100 mg) was added. After 5 minutes the reaction was diluted with 5 mL dichloromethane and extracted twice with water. The dichloromethane was dried and evaporated to yield 20 mg of activated ester.

1-3"-((N'-6-Amidohexyl-2',3'-dideoxycytidine,5'-O-triphosphate)-succinoyloxypropyl)-1'-(3'''-hydroxypropyl))-benzoxazolmonocarbocyanine

The activated ester (2 mg) was dissolved in 200 μL 50% DMF/water and 4 μmol of 6-aminohexyl-ddCTP in 500 μL 0.1 M carbonate buffer, pH 9 was added. After 45 minutes the reaction was terminated. The product was purified by C18 HPLC (3.9 x 150 mm Novapak, 0-75% acetonitrile/ammonium phosphate, pH 6. Yield: 584 nmol. UV/vis 484, 272 nm.

The material was incorporated by a DNA polymerase in a standard sequencing assay, terminating chain extension.

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   (e) US 5,558,991; 9/96, Trainor.

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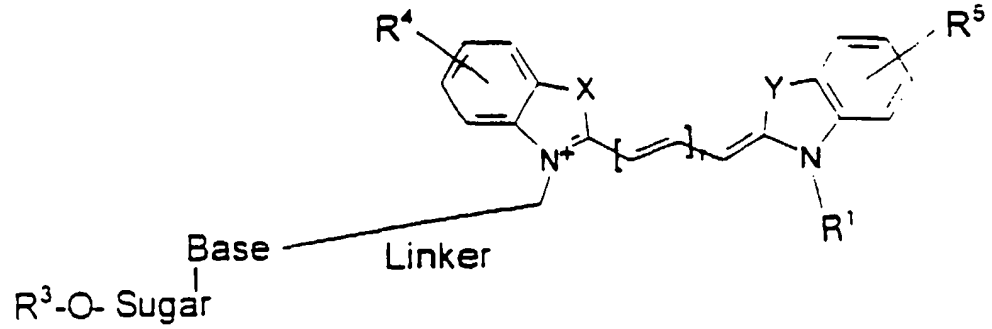
CLAIMS

We claim:

1. A chemical compound of the following formula:



wherein:

R^1 is selected from the group consisting of alkyl, aralkyl, and substituted alkyl groups;

R^3 is selected from the group consisting of H, PO_3^{-2}; $P_2O_6^{-3}$; $P_3O_9^{-4}$, α-thio phosphates; and αBH_3^- phosphates;

R^4 is selected from the group consisting of H, lower alkyl, acyl, $(CH_2)_pCOO(CH_2)_qCH_3$ wherein p is an integer from 0 to 4 and q is an integer from 0 to 4, and 5,6; 6,7; or 7,8-butadienyl;

R^5 is selected from the group consisting of H, lower alkyl, acyl, $(CH_2)_pCOO(CH_2)_qCH_3$ wherein p is an integer from 0 to 4 and q is an integer from 0 to 4 and 5,6; 6,7; or 7,8-butadienyl;

r is 1, 2, or 3;

X or Y are selected from the group consisting of O, S, $C(R^6)_2$, $N(R^6)$, wherein R^6 is CH_3 or a lower alkyl;

and R^3-O-Sugar-Base is a nucleoside or nucleotide.

2. The compound of claim 1 wherein R is selected from the group consisting of substituted alkyl chains, wherein the substitution is OR^2, $COOR^2$, NR^2R^2, or SR^2, wherein R^2 is H, a removable protecting group or a lower alkyl group.

3. The compound of claim 2 wherein R^2 is selected from the group consisting of H, a removable protecting group, or a lower alkyl group.

4. The compound of claim 1 wherein R is $(CH_2)_3OH$.

5. The compound of claim 1 wherein R is selected from the group consisting of $(CH_2)_5COOH$, $(CH_2)_3NH_2$ and C_2H_5.

6. The compound of claim 1 wherein R^3 is selected from the group consisting of PO_3^{-2}, $P_2O_6^{-3}$ and $P_3O_9^{-4}$.

7. The compound of claim 1 wherein the linker is selected from the group consisting of propyl-O-PO_2-O-hexyl, propyl-O_2C-ethyl-CO, propyl-O_2C-ethyl-CONH-hexyl, and propyl-O_2C-ethyl-CONH-propynyl.

8. The compound of claim 1 wherein the linker is between 3 and 25 atoms in length.

9. The compound of claim 1 wherein X and Y are $C(CH_3)_2$.

10. The compound of claim 1 wherein the nucleotide or nucleoside formed by R^3-O-Sugar-Base is selected from the group consisting of IDC-rCTP, IDC-dCTP, IDC-ddCTP, ITC-ddCTP, ITC-ddATP, IDC-dATP, IMC-c7-ddGTP, and OMC-ddCTP.

11. The compound of claim 1 wherein R^3 is selected from the group consisting of PSO_2^{-2}; $P_2SO_5^{-3}$; and $P_3SO_8^{-4}$.

12. The compound of claim 1 wherein R^3 is selected from the group consisting of $P(BH_3)O_2^{-2}$, $P_2(BH_3)O_5^{-3}$, and $P_3(BH_3)O_8^{-4}$.

13. A non-sulfonated carbocyanine dye linked to a nucleotide or nucleoside.

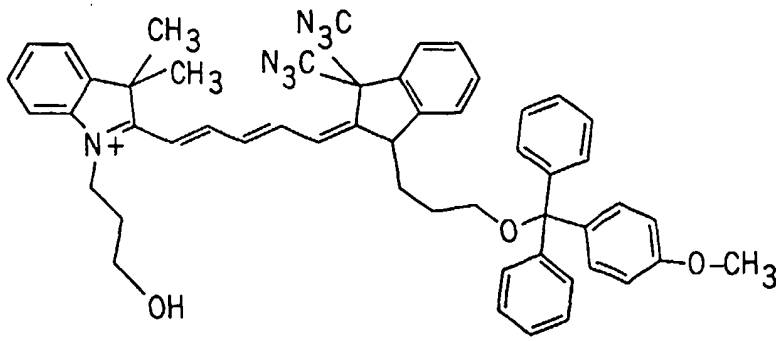
14. The dye of claim 13 wherein the dye is a indocarbocyanine dye.

15. A method of labeling a nucleic acid molecule comprising the step of incorporating the compound of claim 1 into a nucleic acid chain.

16. The method of claim 15 further comprising the step determining the nucleic acid sequence of the molecule.

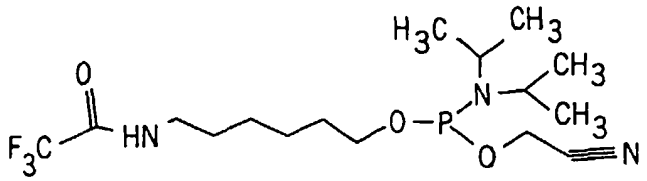
17. A method of labeling a nucleic acid molecule comprising the step of incorporating the compound of claim 13 into a nucleic acid chain.

18. The method of claim 17 further comprising the step of determining the nucleic acid sequence of the molecule.

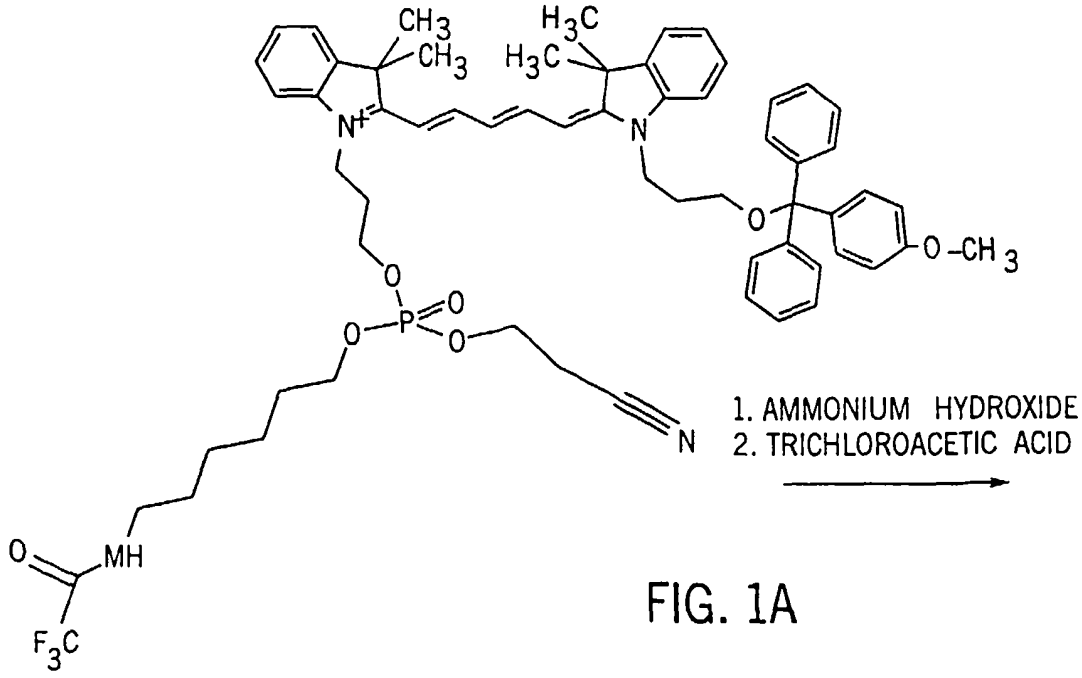


INTERMEDIATE FROM
AMIDITE AYNTHESIS

+



TETRAZOLE
IODINE /
PYRIDINE /
WATER



1. AMMONIUM HYDROXIDE
2. TRICHLOROACETIC ACID

FIG. 1A

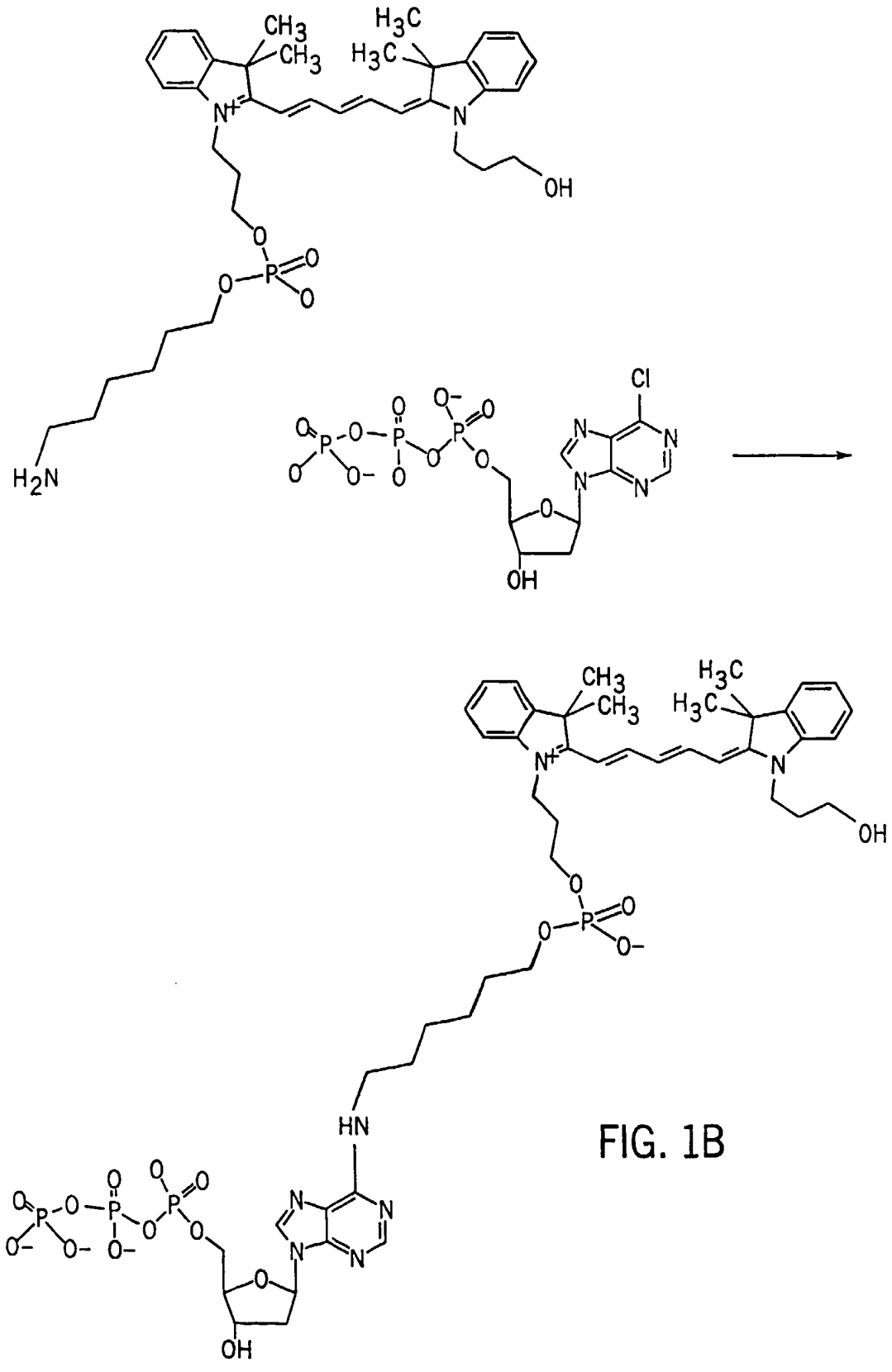


FIG. 1B

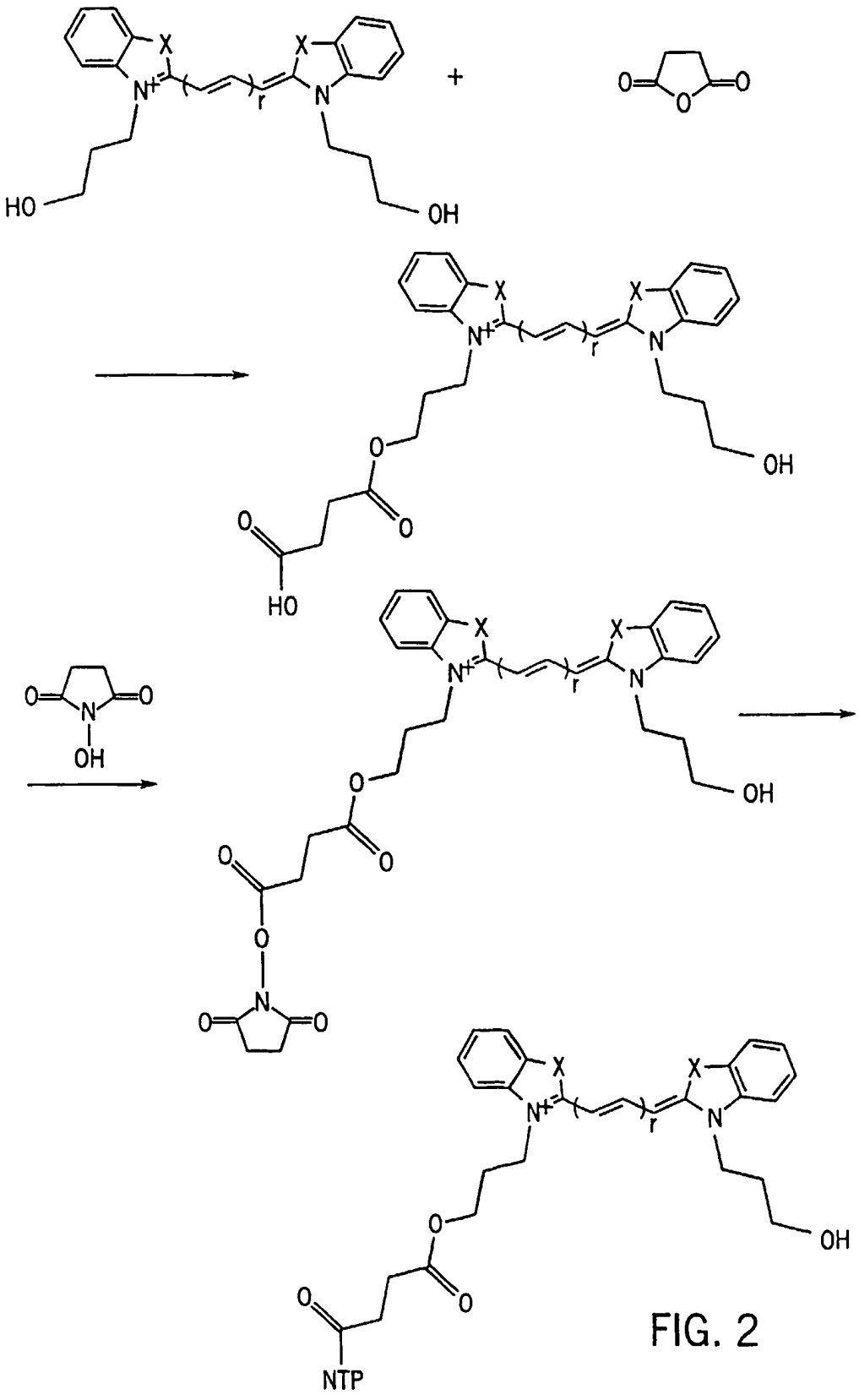


FIG. 2

FIG. 3

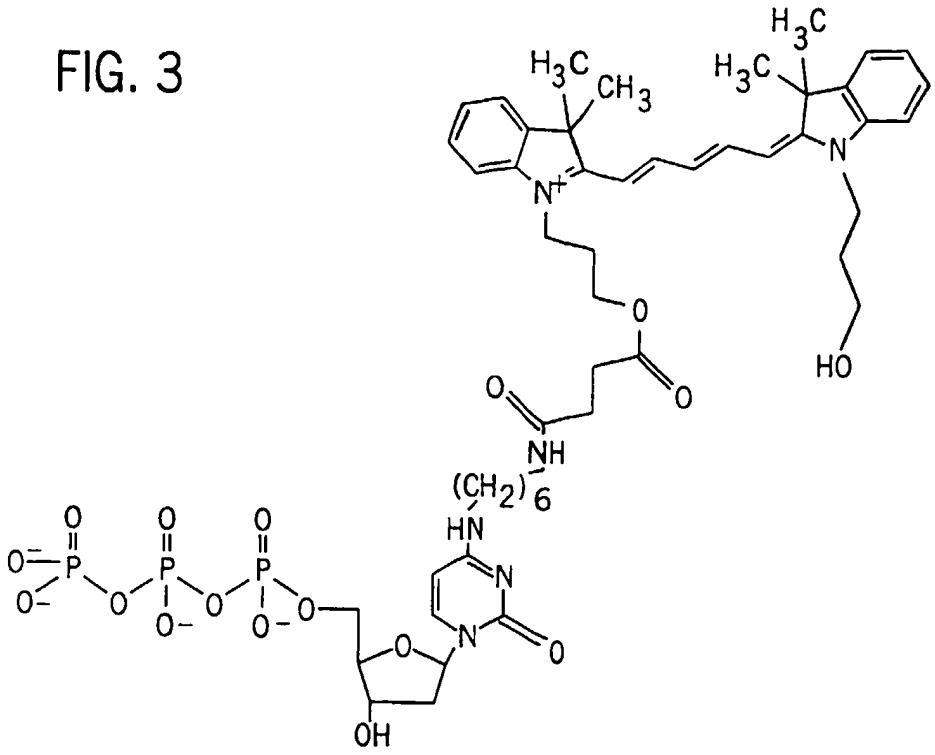
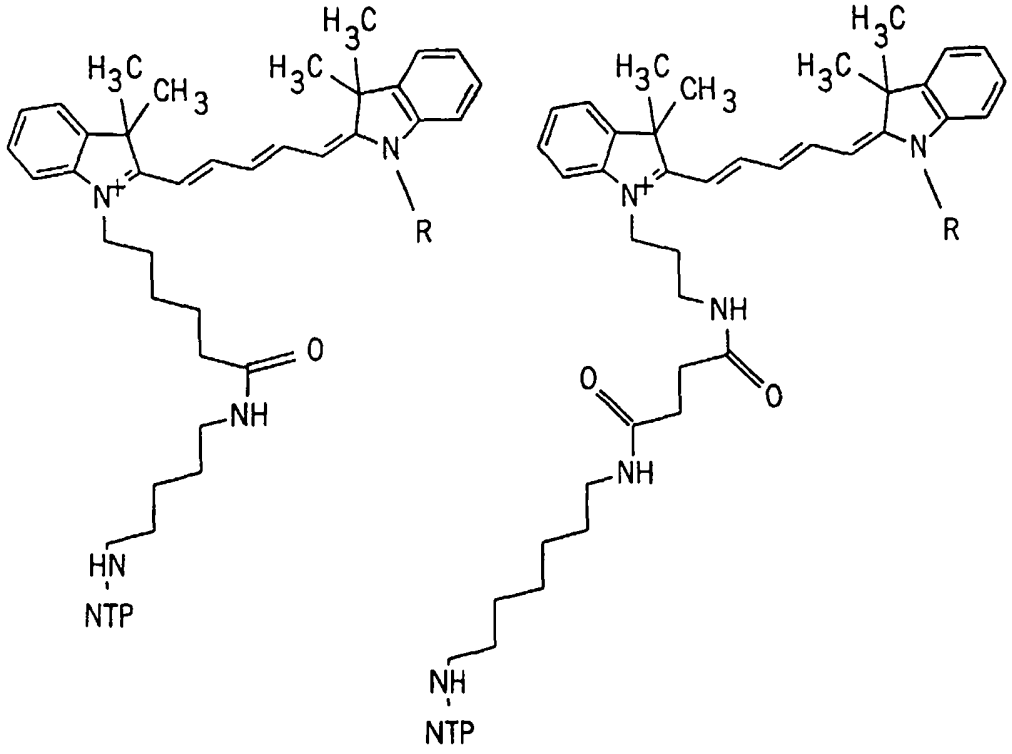


FIG. 4



TWO ALTERNATIVE LINKERS

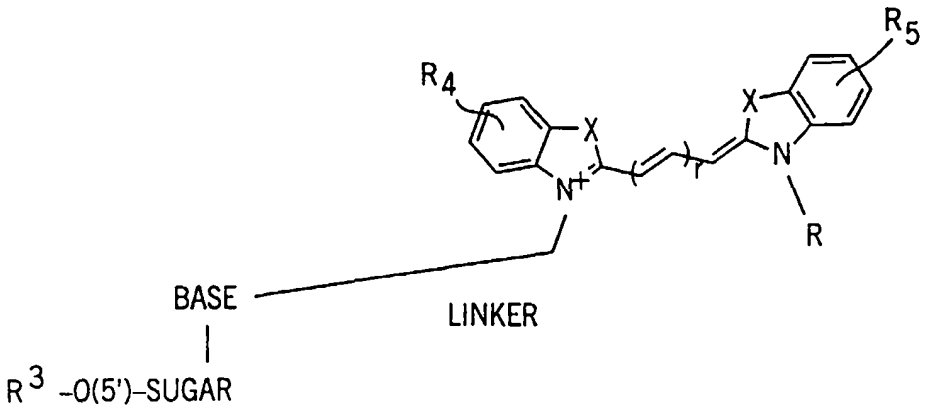


FIG. 5

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/12593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H19/04 C07H19/20 C07H19/10

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	WO 96 22298 A (PHARMACIA BIOTECH INC) 25 July 1996 see claims 1,15-17; figure 3 ---	1-18
Y	US 5 556 959 A (BRUSH CHARLES K ET AL) 17 September 1996 cited in the application see claims 1,9; figures 4,6 ---	1-18
Y	Z. ZHU ET AL.: "Directly labeled DNA probes using fluorescent nucleotides with different length linkers" NUCLEIC ACIDS RES., vol. 22, 1994, pages 3418-22, XP002074425 see abstract; figure 1 --- -/--	1-18

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

' Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
13 August 1998	28/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax. (+31-70) 340-3016	Bardili, W

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/12593

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	J.B. RANDOLPH AND A.S. WAGGONER: "Stability, specifity and fluorescence brightness of multiply-labeled fluorescent DNA probes" NUCLEIC ACIDS RES., vol. 25, no. 14, 15 July 1997, pages 2923-29, XP002074426 see abstract; figure 1	1-18

1

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INTERNATIONAL SEARCH REPORT

...information on patent family members

Inter ...nal Application No
PCT/US 98/12593

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9622298 A	25-07-1996	AU 4749796 A	07-08-1996
		CA 2210900 A	25-07-1996
		EP 0804446 A	05-11-1997
		JP 10504974 T	19-05-1998
US 5556959 A	17-09-1996	NONE	